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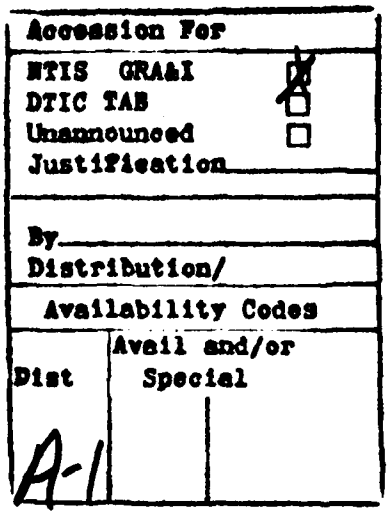

Quantitation of Alkaline Phosphatase Isoenzymes

Using Agarose Containing Wheat Germ Lectin

A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in at Virginia Commonwealth University.

By

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INTRODUCTION

Alkaline phosphatase (AP), [EC 3.1.3.1; orthophosphoric monoester phosphohydrolase is present in almost all tissues of the human body and has long been recognized by physicians as an indicator of bone and/or liver disease. ~~Table 1~~ lists a wide range of tissues which have AP in their cells. Alkaline phosphatase in plasma originates primarily from liver, bone, intestine and placental tissues. These different tissues enzymes are all membrane bound and in the presence of magnesium ion will hydrolyze phosphate monoesters at an alkaline pH (1). This common property gives them the name isoenzyme because it refers to an enzyme from different origins or forms with the same enzymatic activity. The term "isoenzyme", as defined by the International Union of the Biochemistry Commission on Biochemical Nomenclature (2), refers to different forms of an enzyme catalyzing the same substrate and all having genetically different primary structures. Structural differences due to post translational modification are referred to as isoforms.

Genetically, AP isoenzymes arise from three different gene loci; therefore, only three forms are true isoenzymes

Table 1 Organ Distribution of Human Alkaline Phosphatase

Organ	Cell	Relative Activity (per gram of tissue)
Placental	Syntrophoblast Anterior Surface increasing with developement	14.0
G.I. Tract	Ileal	11.0
	Gastric Mucosa	0.2
	Colonic	1.0
Bone/Cartilage	Osteoblast Chondroblast	3.0
Kidney	Proximal, distal tubules Henle Loop: at brush border	2.5
Liver	Sinusoid Hepatocyte -strongest concentration in areas closes to portal vein	2.5
Spleen	Not defined	0.3
Lung	Not defined	0.3
Marrow/ Blood	Mature Neutrophils	0.02
Pancreas	Acinar	0.03
Parotid	Acinar	<0.01

(3). Liver, bone and kidney are products of one gene and therefore are isoforms of each other. Intestinal AP arises from a second gene locus and placental AP originates from a third gene locus. Once these isoenzymes are coded for and produced, they undergo post translational modification (4) including the addition of terminal sialic acid residues and glycosylations. Sialic acid accounts for the net charge differences between AP isoenzymes (5). Post translational glycosylation occurs through the addition of carbohydrate moieties and is thought to account for the differences between the bone, liver and kidney AP (6).

All of the AP isoenzymes cleave the same "in-vitro" substrates allowing the total serum alkaline phosphatase to be determined. However, the specific "in-vivo" substrates and the exact function of each isoenzyme is not fully known. Inside the cell the AP isoenzymes are thought to perform specific functions such hydrolysis and synthesis of phosphate esters, and the regulation of certain intracellular processes including the maintenance of phosphoryl metabolites in a steady state. Since AP is bound to the cell membranes, it is also thought to function in the active transport of inorganic phosphate, calcium, lipids, and proteins (7).

There are a wide variety of diseases (see Table 2) that increase AP activity including hepatic disease where the major rise is seen in cholestatic involvement from any cause, and where the total AP may be greater than twice

Table 2 Disease Associated with an Elevation in Total Alkaline Phosphatase

1. Hepatic Disease - Major rise (>2 time normal) reflects cholestatic involvement from any cause.

    - taken in context, less elevation occurs in primary hepatocyte disease and only in later stages as a possible co-involvement with canalicular obstruction.

    - the phenomenon of discordant rise of alkaline phosphatase with no or minimal rise of bilirubin.

    - liver I and biliary (liver II) are the major isoenzymes increased.

2. Bone Disease - derived from osteoblast

    - active osteomalacia.

    - Paget's disease, osteogenic sarcoma, neoplasms.

    - Bone AP is the major isoenzyme increased.

3. Renal Disease - renal failure especially if tubule involved.

    - in association with renal failure.

    - possible changes primarily associated with hemodialysis.

    - bone and intestinal AP are the major isoenzymes increased.

4. Pancreatic Disease - primary involvement with obstruction of pancreatic duct, and secondary if common bile duct involved.

    - liver I AP is the major isoenzyme increased.

5. Endocrine Disease

    Thyroid - hyperthroidism with concurrent bone disease.

    Parathyroidism - hyperparathyroidism.

6. Neoplastic disease

Primary - ectopic forms, Regan.

- liver I and AP variants are the major isoenzymes increased

Secondary - metastatic lesions in bone and liver.

- bone, liver I and biliary isoenzymes are the major isoenzymes increased.

7. Reflecting Vascular Proliferation

Following infarcts of the heart, lung, kidney and spleen.

- liver I AP is the major isoenzyme increased.

8. Gastro Intestinal Disease

Extensive mucosal damage to small intestine, small bowel infarction.

- liver I AP is the major isoenzyme increased.

9. Induction with Drugs

- liver I AP is the major isoenzyme increased.

10. Hematological Disease

Leukemia and lymphoma either primary association with tumor or reflecting infiltration at various sites.

- liver I and biliary isoenzymes are the major isoenzymes increased.

the normal AP activity level. Increased AP is also associated with conditions where there is increased osteoblastic activity such active osteomalacia and Paget's disease (8).

AP found in the liver is attached to both the hepatic sinusoidal membrane and the bile canalicular membrane of the hepatocyte (9) and occurs in two forms. There is a soluble liver isoenzyme, associated with the sinusoidal membrane, called liver I AP which makes up most of the total AP in normal serum.

Elevations of liver I can occur in liver, pancreatic, cardiac, endocrine and neoplastic diseases. The second liver form, commonly called biliary AP (or liver II) is a membrane bound species found in plasma only under pathological conditions involving the canalicular membrane (10). The biliary AP circulates in plasma attached to membrane fragments and has a large molecular weight (2,000,000 Daltons). Liver I AP when released from the sinusoidal membrane, normally appears in the plasma, whereas biliary AP (liver II) bound to the canalicular membrane does not and is excreted with the bile. In patients suffering from cholestasis and related pathological conditions fragments from both the sinusoidal and canalicular membranes enter the circulation and account for the appearance of both forms of liver AP (11). Patients with cancer may typically show large elevations of the biliary isoenzyme which is a sensitive marker for

the identification of liver metastases (12). Viot, et al., showed that biliary AP, while not specific for cancer, is of value in diagnosing active cancer in high prevalence populations (13).

Shinhai and Akedo demonstrated that biliary AP enters the circulation through increased permeability of hepatocyte tight junctions in cholestasis (14). The increased of AP seen in cholestasis, has been demonstrated to be due to an increase in the "de novo" synthesis of AP and not just an increase in cellular release of the enzyme (15-16). Hatoff and Hardison (17) found that the cause of the increased enzyme production during cholestasis was due to the presence of bile acids which they showed would increase AP in rat liver cell cultures. The concentration of bile acids needed to increase AP is similar to that found in plasma during cholestasis. Therefore, the degree of AP increase is thought to be related to the hepatic content of bile acids. One theory states that during cholestasis the following events may occur:

1) obstruction induces increased enzyme production.
2) leaching of enzymes from membrane surfaces, possibly by bile salts.
3) shedding of membrane fragments through bile salt actions.
4) escape of unbound (soluble) and membrane bound enzymes through leaky tight junctions of the hepatocyte.

Bone AP participates in the bone calcification and or recalcification process of the osteoblast. Bone AP has been shown to hydrolyze pyrophosphate which is an inhibitor of bone mineralization (18). However, the exact nature of its role is not understood. It is known that increased bone AP is seen during increased calcification or osteoblastic activity. Increased osteoblastic activity occurs during normal bone remodeling and in response to pathological bone destruction. During bone remodeling, there is bone resorption followed by bone formation (19). This process is under hormonal control which causes phagocytes and osteocytes to cluster together to form osteoclasts. The osteoclasts begin to locally reabsorb calcium and the bone matrix resulting in a cavity in the bone. During the process of bone re-formation, preosteoblasts are attracted to the bone cavity by the release of local factors. Once present, the preosteoblasts mature and start laying down osteoid which is collagen and matrix. This process is designed to replace bone removed in the resorption step (20). Once the osteoid reaches a certain thickness, mineralization occurs. Before puberty, the process of bone formation is greater than resorption; therefore, skeletal mass, osteoblastic activity and bone AP are high. In adulthood, the rate of formation and resorption is in equilibrium until after age 50 when bone resorption exceeds formation and the total body bone mass begins to decrease. It is interesting to note that bone AP

begins to increase after age 50 probably in response to the resorption process (7).

Besides AP's role in normal bone remodeling, there are a number of diseases that cause increased bone AP. Some of these conditions, such as osteoporosis, are due to hormonal problems. Osteoporosis occurs most frequently in women in the $\geq$50 years of age group and is thought to be related to the decline in estrogen levels seen in postmenopausal women. These women often experience spontaneous fractures of the spinal vertebrae, hips and forearms. The cause of these fractures is due to bone loss. The exact role of estrogen in preventing this bone loss is not understood. Riggs and his group at the Mayo Clinic have suggested that estrogen may directly stimulate osteoblasts which are the bone forming cells (21). Currently, estrogen is given as a treatment for osteoporosis, but it has been shown not to be effective if given after the 8 to 10 year period following menopause. Estrogen therapy for osteoporosis can have side effects including cancer of the endometrium and blood clots. These two complications prevent the routine use of estrogen prophylaxis therapy in asymptomatic women.

Osteomalacia is a decrease in mineralized bone mass in which not mineralized matrix or osteoid is present in excess. Since a large proportion of osteoid tissue is not calcified the bones are soft. The typical diagnostic pattern seen in osteomalacia is slightly reduced plasma

calcium, greatly reduced plasma phosphate, and elevated parathyroid hormone.

Paget's disease is a chronic bone disorder that is characterized by bone destruction with resorption and replacement with poorly mineralized osteoid tissue and variable amounts of fibrous tissue. Bony enlargements of variable size that are soft, structurally weak, and deformed with stress are seen most often in weight bearing bones of men after age 30. The highest elevations of serum AP are seen in patients with Paget's disease.

Intestinal AP, unlike liver or bone which are isoforms of each other, is an individual gene product. This isoenzyme can be found in up to 30% of the total AP in blood group O and B individuals who secrete soluble proteins into body fluids such as saliva (22). However, Warnock demonstrated through special staining techniques that intestinal AP can be isolated from all groups after the ingestion of a fatty meal (23). The duodenal epithelium, where the intestinal absorptive capacity is the highest, has been found to have the highest intestinal AP activity (24). The function of intestinal AP is not completely understood. There is some evidence that intestinal AP transports fatty acids and that it is involved in the absorption of calcium and phosphate. Serum intestinal AP does increases in conditions where there is extensive mucosal damage to the intestine and stomach (24).

Total AP elevations can be seen in "normal"

conditions such as pregnancy. The AP that increases during pregnancy is of placental origin. Placental AP is located on the outer most surface of the syntrophoblastic membrane and begins to increase towards the end of the first trimester of pregnancy and continues to increase until the end of the third trimester (25). During this increase, placental AP levels will be twice the concentration observed in non-pregnant women. The level of placental AP parallels the maturity of the cytotrophoblast of the microvilli. Placental AP may remain elevated up to a month after delivery of a viable fetus. Placental AP is extremely heat stable and its enzymatic activity is inhibited by the amino acid phenylalanine (26).

A form of AP that is indistinguishable from placental AP is called the Regan isoenzyme (26). Like placental AP, it is inhibited by phenylalanine and is heat stable. The Regan isoenzyme is a rare allelic form of placental AP and is the principle AP produced by HeLa cells, a cultured line of human carcinoma cells (27). The Regan isoenzyme has been demonstrated in bronchogenic carcinoma, breast cancer and carcinoma of the colon. The presence of the Regan isoenzyme represents a derepression of the gene coding for placental AP in the cancer cell (28).

Another AP isoenzyme variant has been identified in a variety of diseases bound to immunoglobulin G. This complex is recognized as a slow migrating band on PAGE due to the increased molecular weight of the immune complex

(29). Treatment of the complex with papain has shown that the AP is attached to the antibody on the variable region (30) which demonstrates that the complex is formed by an antigen-antibody reaction between liver or bone AP and IgG antibodies.

The isoenzymes discussed above can be identified in the serum when present in normal or abnormal amounts. Normal human serum contains the liver I and bone isoforms and may contain intestinal isoenzymes in some individuals. Table 1 shows the tissue sources of AP. Kidney AP is secreted into the urine and does not appear in serum. The other organs contribute only minor amounts of enzyme to the total serum AP. Since the isoenzymes retain their specific properties when released into the circulation, methods to separate, identify and quantify the fractions causing the elevations provide clinically useful information on the pathologies involved.

Identification of AP isoenzymes has been based on variations in stability to heat (31), response to inhibitors (32-34), and electrophoretic mobility at an alkaline pH (35)(36). In most cases these separation techniques are employed only to differentiate bone from liver AP.

Heat stability has been used as a simple means to differentiate liver from bone AP. This test is performed by heating an aliquot of the patient's serum to 56°C for 10 minutes. A total AP is then performed on the heat

treated aliquot along with a not heated portion of patient's serum. The activity of bone AP is reduced when heated. Epstein, et al., noted that total AP of less than 20% after heating indicates that the AP elevation is probably due to the bone isoform (7). The liver and intestinal isoenzymes also lose some activity after warming to 56°C for 10 minutes but are more heat stable than bone AP. Total remaining AP activity of 22 to 55% after heating is probably due to the liver fraction. Since bone, liver and intestinal isoenzymes are all heat labile, heat stability alone, is not adequate to distinguish these species and is used in conjunction with other separation procedures.

Urea inactivates AP by interfering with hydrogen bonding as well as weakening hydrophobic interactions among the proteins. Since the hydrogen bonding and hydrophobic interactions among the various isoenzymes will vary, this method has the potential to differentiate them. Birkett and co-workers demonstrated that the order of inhibition with urea is bone > liver > intestinal > placental (32). Besides urea, phenylalanine and homoarginine can be used to inhibit the AP fractions. Phenylalanine inhibits intestinal and placental isoenzymes but not the liver or bone fractions. In contrast, homoarginine inhibits liver and bone AP but not the intestinal or placental isoenzymes (33). Urea and amino acid inhibition has been applied in combination with heat

stability as a means to separate the AP fractions. None of these inhibition procedures give unambiguous results.

Other means to separate and quantitate AP isoenzymes take advantage of post translational modifications. Glycosylations of the AP isoenzymes occur after the gene product is formed. Sialic acid residues are present on the carbohydrate moiety of the glycoproteins on the different AP fractions. Sialic acid residues impart a negative charge on the isoenzyme surface. Kamoda and Sakagishi found that the amount of sialic acid varies among the different isoenzymes (37). This variability has been used as a means to separate the isoenzyme fractions using neuraminidase (sialidase) followed by electrophoresis on acrylamide gel. Neuraminidase will retard the mobility of bone and liver AP. The mobility of intestinal AP which doesn't contain sialic acid is unaffected. The disadvantage of neuraminidase incubation is that the incubation period must be brief and controlled to maximize separation between liver and bone. Prolonged neuraminidase incubation may cause complete removal of sialic acid residues resulting in equal mobilities on acrylamide electrophoresis.

The physical differences between the isoenzymes of AP such as carbohydrate content, net charge, and molecular weight all affect their migration in an electric field. Electrophoresis using agarose gel and cellulose acetate separate the isoenzymes soley on the basis of their net

charge. The charge difference between AP isoenzymes is small especially between the bone and liver fractions; therefore, on agarose and cellulose acetate, separation of these isoenzymes is poor (36).

Discontinuous polyacrylamide gel electrophoresis (PAGE) is a high resolution technique which separates proteins based on both size and charge (38). Individual isoenzymes arrange themselves in the loading and stacking gels into thin, discrete, starting zones in order of decreasing mobilities. The isoenzymes arranged in this order will enter the separation gel and migrate according to their electrophoretic mobilities and molecular size. Unfortunately, the resolution between liver and bone isoenzymes using PAGE is not adequate to allow densitometric quantitation. AP are qualitatively interpreted for the presence or absence of individual isoenzyme bands.

Isoelectric focusing for the separation of AP has gained interest in recent years. This technique takes advantage of the zwitter ion property of the proteins (39). The medium used (usually polyacrylamide or agarose) contains low molecular weight amphoteric molecules with varying isoelectric points (pI). For AP separation, an agarose pI gradient of 3 to 8 is usually applied. The passage of the current prior to the addition of the sample results in the formation of a stable pH gradient in the gel. The proteins (sample) placed in the pH gradient will

migrate through the gel until they reach their specific isoelectric points, where the charge on the protein is zero and it stops migrating. Isoelectric focusing provides the best resolution of AP isoenzymes of any form of electrophoresis. Unfortunately, the many bands that result (up to 12 for AP), are difficult to interpret with respect to specific tissue origins. For example 4 to 6 bands are seen for the bone isoform alone. Isoelectric focusing has not been widely accepted in the routine clinical chemistry laboratory.

Wheat germ lectin (WGL) has been demonstrated to separate bone and liver AP by both solution precipitation and electrophoretic techniques (36)(40). WGL is a dimeric protein with each polypeptide chain containing two binding sites for the N-acetylglucosamine residues found mainly on the bone fraction (41). WGL binding also changes the net charge of the bone isoenzyme and thus affects its electrophoretic mobility and can produce precipitation of the isoenzyme under appropriate conditions. Schreiber and Whitta (40) described the electrophoretic separation of AP isoenzymes using WGL in agarose. Using WGL electrophoresis, the order of migration is liver I > Intestinal > biliary (Liver II) > Bone (see Figure 1). Biliary AP (liver II) if present, migrates just anodally to the bone fraction and obscures quantitation of either isoenzyme. Parallel electrophoresis on agarose gel without WGL allows complete separation of biliary AP from other

Figure 1

Electrophoretic mobility of alkaline phosphatase isoenzymes on agarose gel with and without wheat germ lectin versus relative fluorescence. Migration is from cathode to anode.

fractions and permits subtraction of the biliary from the bone/biliary unresolved band. Intestinal AP migrates cathodally to the liver I band and is well separated from liver and bone bands. Densitometry of the separated and stained AP isoenzyme fractions permits quantitation of the individual species present in serum.

I report on the analytical and clinical validation of the quantitative alkaline phosphatase isoenzyme procedure using wheat germ lectin agarose electrophoresis. The study of WGL electrophoresis was initiated by Dennis Jay, Ph.D., while a postdoctoral fellow in this laboratory. Dr Jay discovered that the sensitivity of the procedure could be improved through the use of the substrate 4-methyl umbelliferyl phosphate in the method previously reported by Schreiber and Whitta (40). Dr Jay used this modified WGL procedure to determine method linearity, minimum detectability, inter-assay precision on 2 frozen serum pools, and reference limits for females. His data is reported in this thesis (and acknowledged in each instance) to provide a complete analytical and clinical evaluation of the procedure.

MATERIALS AND METHODS

Specimens

All blood was collected in evacuated glass tubes without anticoagulant and allowed to stand 1-2 hours while clotting occurred. Each clotted specimen was centrifuged at 1500 g at room temperature and the serum transferred to polypropylene vials. If analysis could not be performed within a few hours, serum was stored frozen at -20°C.

Specimens collected for reference ranges were from apparently healthy, non-fasting blood donors at the Richmond Metropolitan Blood Service. All donors were asked to complete a questionnaire about illness history, medication use and recent fractures.

Specimens for method comparison studies were obtained frozen from the electrophoresis section.

Clinical correlation specimens were obtained from the chemistry profiles and stored frozen within 24 hours of collection. Total AP activity had to be > 150 U/L to be included in this study.

AP Activity

Total AP enzyme activity was measured using the International Federation of Clinical Chemistry method proposed by Tietz, et al (42). The conversion of p-

nitrophenyl phosphate to p-nitrophenol, at 37°C, pH=10.4, was monitored at 405 nm using a Cobas-Bio centrifugal analyzer (Roche Diagnostics Systems, Nutley, NJ). The activity of each AP isoenzyme was calculated by multiplying the total AP activity times the fractional percentage from densitometry of the electrophoretically separated and stained AP isoenzymes.

Polyacrylamide Gel Electrophoresis

PAGE was conducted in 7 x 63 mm glass tubes containing 7% separation gel, Tris-chloride buffer pH = 8.7, 3% stacking gel, Tris-chloride buffer pH = 7.0, 2.7% loading gel, Tris-chloride pH = 6.8. The electrophoresis tank buffer is Tris-borate at pH 8.8 (39). An aliquot of each sample is heated to 56°C for 10 minutes and run in parallel with the not heated sample. The stain substrate was prepared by adding 0.023g of 5-Bromo-4-chloro-3-indolyl phosphate to 10 ml ofN,N Dimethyl formamide. The stain buffer consists of 14.15 g of Tris (hydroxymethyl) amino methane and 0.51 g of magnesium chloride in 500 ml of distilled water. The stain solution was prepared by mixing 6 ml of stain substrate with 45 ml of stain buffer and 45 ml of water. Each gel was removed from the tube and placed in a tube containing 5 ml of stain solution and allowed to incubate for 2 hours at 37°C. After 2 hours the stain solution was removed and each tube was filled with 7% acetic acid stopping further color development.

Wheat Germ Lectin (Triticum vulgaris)

Highly purified, essentially salt free Triticum vulgaris was purchased from Sigma Chemical Co., St Louis, Mo., L-9640. To make the working lectin reagent, dissolve WGL in distilled water to a concentration of 10 g/L. Aliquot 0.4 ml of this working solution into individual polypropylene vials and store at -20°C. According to the manufacture, WGL rehydrated and stored in this manner will last indefinitely. We have found that full activity is maintained for at least 6 months. Each lot of WGL must be pretested for suitability in this procedure (see discussion).

Triton X-100

Triton X-100 was purchased from Sigma (T-6878). A working solution of triton X-100 was prepared by diluting the stock solution 1:10 with distilled water.

Electrophoresis Buffer

Trizma (50 mmol)/barbital (14 mmol)/sodium barbital (50 mmol) buffer at pH 8.9 is available prepackaged from Sigma (710-1). Dissolve one 10 g vial in distilled water to make one liter of buffer and if necessary adjust to pH 8.9 $\pm$ 0.1 . The working buffer must be stored at 4°C and the pH rechecked weekly.

AMP-Metal Ion Buffer

The stock metal ion solution was prepared by dissolving per liter: 112.2 mmol (4.266 g) N-hydroxyethylene-diaminetriacetic acid (Sigma H-2378), 56.1 mmol (1.613 g) of ZnSo4 7H2O, (Fisher Z-68), 112.2 mmol

(2.407 g) magnesium acetate 4H2O (Aldrich 22,864-8). Dissolve the N-hydroxyethylene-diaminetriacetic acid in 700 ml distilled water. Add the zinc sulfate and allow to dissolve completely. Add magnesium acetate, allow to dissolve, and dilute solution to 1 liter. The solution is stable for three months.

AMP-metal ion buffer is prepared by adding 393 mmol (17.52 g) of 2-amino-2-methyl-1-propanol, (AMP; Kodak, 4780) to 400 ml of distilled water and adjusting to pH 10.40 ($\pm$0.05) at room temperature. 10.0 ml of the metal ion solution is added slowly with stirring to the AMP buffer. Adjust the pH to 10.40 and dilute to exactly 500 ml with distilled water. The AMP metal ion buffer must be stored refrigerated in a glass container and is stable for three months.

Agarose Gel

Agarose powder Type I, Low EEO was from Sigma (A-6013).

4-Methyl Umbelliferyl Phosphate Substrate

The substrate 4-methyl-umbelliferyl phosphate Sigma (M-8883) was prepared by weighing out 6.0 mg of the substate into a 15 ml red top tube. Add 11.5 ml of the AMP-metal ion buffer and mix by inversion or vortex. This amount is enough substrate to stain 2 gels (5 ml per gel). The substrate solution should be prepared and used fresh.

Agarose Gel Support Film

GelBond™ film (53734; 85 mm x 100 mm) purchased from

FMC Corp., Rockland, ME.

Agarose Gel Preparation

Separation and quantitation of bone, liver I, biliary (liver II) and intestinal isoenzymes requires the use of agarose gel with and without lectin. Place 0.10 g of agarose powder into the bottom of a 15 ml red top evacuated tube with a spatula to minimize agarose powder clinging to the upper part of the tube. Each 0.10 g of agarose will prepare one gel. Slowly pipet 4.0 ml of distilled water to the bottom of each tube while hand rotating. Rotation of the tube should continue during the addition of all liquids to ensure mixing and to prevent clumping of the agarose. Add 2.0 ml of the triton X-100 working solution to each tube, and while still rotating add 4.0 ml of the electrophoresis buffer to each tube.

Loosely replace the stopper into each tube to allow trapped air and water vapor to escape. Place each tube into a boiling water bath insuring that the water level in the bath is below the mouth of all the tubes. A stirring bar may be used to prevent tubes from bumping together. Complete dissolution of the agarose takes 10 minutes. Gently spin each tube immediately upon placing in the bath and frequently through out the boiling period. During this time do not invert the tubes because undissolved agarose will adhere to the upper part of the tube. After 10 minutes remove each tube from the water bath and set in a rack at room temperature. There should be no foam at the

liquid air interface and the dissolved gel will appear cloudy. The gel in each tube should be allowed to solidfy undisturbed in a test tube rack. Once the gel is completely cool, it can be capped and stored refrigerated. Gel tubes are stable for three months.

To pour a gel, a tube containing the solidified agarose was removed from the refrigerator and placed into a boiling water bath to remelt. While the agarose tubes are in the boiling water bath, obtain one support film for every gel to be prepared. Each film must be cut to 75 mm x 100 mm in order to give the proper gel thickness after pouring. Each film has a hydrophilic and a hydrophobic side. The molten agarose must be layered onto the hydrophilic side. If poured onto the hydrophobic side, the gel will separate from the film during electrophoresis. The hydrophilic surface of the film can be determined by dropping a small amount of water onto the film. Water will bead on the hydrophobic side and spread out on the hydrophilic side. Place the film on a flat, level surface with the hydrophilic side up. Allow a small drop of water to flow under each corner of the film to secure it to the surface. Repeat this procedure for each gel to be prepared. The hydrophobic side of the films can be labeled with a laboratory marker for identification prior to layering with agarose. When the agarose has just liquified, (approximately 5 minutes) the tubes are removed from the water bath and at that point the agarose should

be at the correct temperature for adding the WGL (approximately 70°C); 0.40 ml of the WGL solution is pipetted into the agarose. The working WGL is stored frozen; it must be thawed and mixed prior to its addition to the agarose. The agarose and WGL are mixed by gentle inversion; caution must be taken not to allow bubbles to form in the tube. If bubbles form they may be removed with a pasteur pipette. The agarose/WGL mixture is immediately poured onto a 75 x 100 mm gel bond film using a constant but slow pouring motion. Hold the mouth of the agarose tube about 3/4 inch above the center of the film. Slowly tip the tube allowing the agarose to flow evenly out onto the film. Pour all the agarose solution into the center of the film. To avoid blemishes and pits in the gel do not allow individual drops of hot agarose to hit the gel. The molten agarose may overflow the film if the solution is poured too rapidly or if poured in segments. Molten agarose without WGL is poured in the same manner as soon as it is removed from the water bath. Cover the gel with a box or lid to prevent air currents from disrupting the gel surface, and allow it to cool for at least five minutes. After cooling, place the gel in an air tight hydrated chamber and store at 4°C overnight. The gel should not be used immediately. Gels will remain stable at least 3 months if kept refrigerated in the hydrated container.

Electrophoresis

Bring all reagents, samples, controls and agarose gels to room temperature. Insure all samples and controls previously frozen are completely thawed and mixed. For each run, obtain a gel with and without WGL from the hydrated chambers and remove excess moisture by blotting the gel surface with blotter paper. Place a Beckman Paragon sample template (pink) onto each gel about 2 cm from the bottom. Slowly roll the template onto the gel thus preventing bubbles from being trapped near the sample slots. Place 10 ul of patient sample or control material onto each slot of the WGL gel and onto the corresponding slot of the agarose without WGL, and allow to sit for 10 minutes. During this time, fill each chamber of the Beckman Paragon electrophoresis apparatus with 45 mls of electrophoresis buffer. At the end of 10 minutes, remove excess fluid from the template using a sample blotting paper. Place each film into a Beckman Paragon gel holder insuring the point of application is placed at the cathode. AP is negatively charged and will migrate towards the anode. Place each gel holder into the Beckman Paragon electrophoretic chamber and allow migration to occur for 45 minutes at 100 volts constant current. The Beckman Paragon apparatus is operated at room temperature without external cooling. Comparable equipment from other manufacturers should give equivalent performance.

Staining and Scanning

During the 45 minute electrophoresis time, prepare the 4-methyl-umbelliferyl phosphate substrate solution. Just prior to the completion of the 45 minutes, add 5 ml of the substrate solution to a separate staining box (90 x 120 mm) for each gel. When electrophoresis is complete, float the film gel side down in the staining box. Weight the gel down in the substrate solution by placing nickels on each side of the film to insure the gel is completely submerged. Close the staining box and place each box in a 37°C incubator for 45 minutes. At the end of the 45 minutes, remove the gels from the staining box and place them in a distilled water bath for five minutes to remove excess substrate. The gels are then ready to visualize under a wood's lamp and scan with a densitometer in the fluorescent mode (we used a Beckman CD-200 densitometer, Beckman Instruments, Inc., Brea, CA).

RESULTS

Alkaline phosphatase isoenzyme mobility using WGL is shown in Figure 1. Liver I AP is not bound by WGL; therefore, it migrates most rapidly to the anode. Intestinal AP, if present, migrates just cathodally to the liver I fraction. Intestinal AP appears as a shoulder on liver I. Bone AP complexes with the WGL in the gel and the migration is retarded such that bone AP remains near the origin. Biliary (liver II) AP if present, will either migrate just anodally to bone or co-migrate with bone AP. This co-migration prevents quantitation of bone or biliary AP when both are present. However, biliary AP is completely separated from the other isoenzymes by electrophoresis on agarose containing triton x-100 but without WGL. Without triton X-100 treatment, biliary AP will migrate with liver I on agarose. Triton x-100 does not affect the mobility of bone or intestinal isoenzymes which co-migrate with liver I AP in the presence of triton X-100 without WGL. Bone AP can be quantitated by subtracting the percentage of biliary (from the agarose without WGL) from the percentage of bone/biliary fraction on agarose with WGL.

Analytical sensitivity and linearity for liver I and

bone AP was determined by analyzing multiple dilutions of a sample with 2000 U/L total AP activity. Figures 2 and 3 show that a linear response was obtained from 6-1200 U/L for liver I AP and 4-800 U/L for bone AP. Lower activities could not be distinguished from zero.

Inter-assay precision of the WGL method was evaluated by separate analysis of five different serum pools. The pools were prepared by mixing sera from patients with normal or elevated amounts of the various isoenzymes. Aliquots were stored at -20 °C prior to analysis. Table 3 presents the precision data for these serum pools. Liver I precision varied from 6-14% CV and bone precision from 8-14% CV among four pools. Bone isoenzyme precision was 17% when measured in one pool containing biliary isoenzyme. Biliary (liver II) precision was 31% CV in one pool and intestinal precision was 26 and 23% in two pools.

Recovery studies were performed to determine that the separation of isoenzymes reflected the tissue origin of the AP species and that the separation was not influenced by the relative amounts of each isoenzyme or other proteins present. For example, a serum with high bone activity was mixed in proportions 100:0, 75:25, 50:50, 25:75 and 0:100 with a serum having normal liver activity. The expected activity in the three admixtures was calculated from the total activity and the isoenzyme distribution in the 100:0 and 0:100 samples. If the expected activity was recovered for the admixtures, this

Figure 2

Linearity for bone alkaline phosphatase and the least amount of bone alkaline phosphatase detected using agarose containing wheat germ lectin.

Figure 2

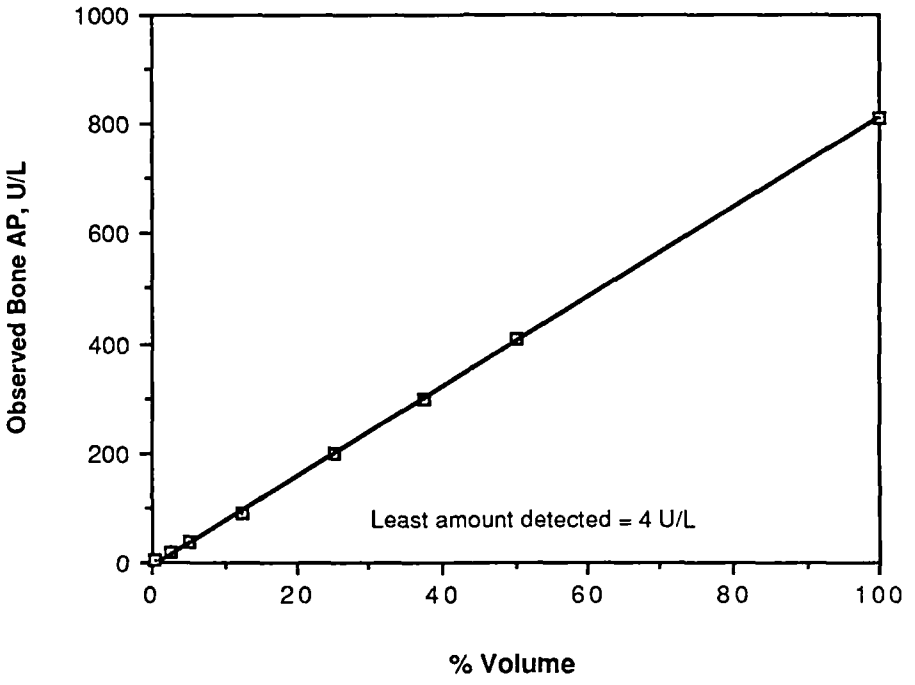
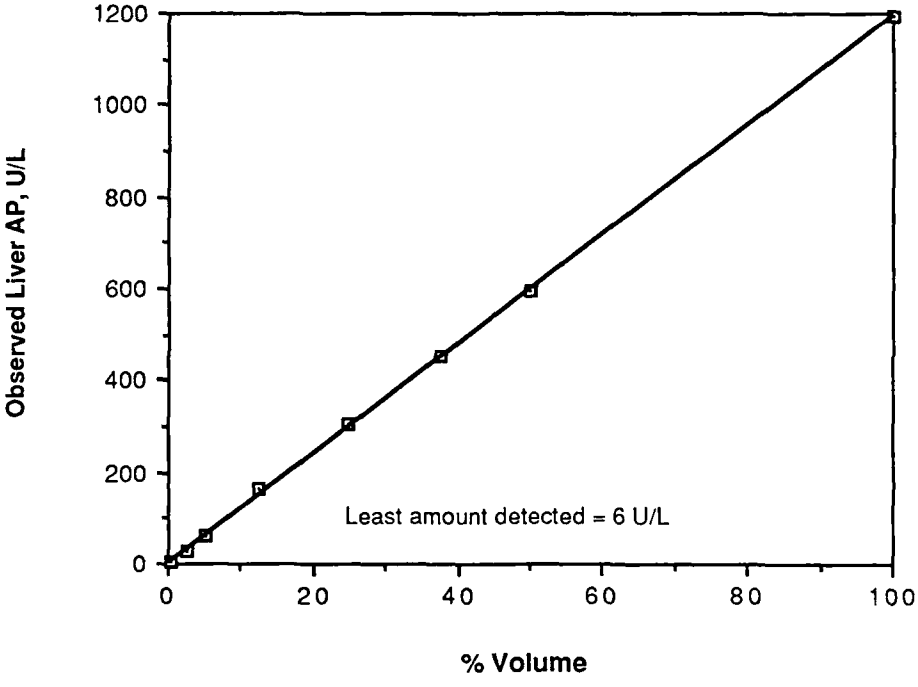

1000
800
600
400
200
0
Observed Bone AP, U/L
Least amount detected = 4 U/L
0
20
40
60
80
100
% Volume

Figure 3

Linearity for liver alkaline phosphatase and the least amount of liver alkaline phosphatase detected using agarose containing wheat germ lectin.

Figure 3


1200
1000
800
600
400
200
0
Observed Liver AP, U/L
Least amount detected = 6 U/L
0
20
40
60
80
100
% Volume

supported that the lectin concentration, electrophoretic, and staining conditions are suitable to prevent any bone isoenzyme from migrating to the liver position. Table 4 reports results for recovery studies under a wide range of serum compositions. Sera from patients with elevations of the different AP isoenzymes were examined as well as patients with renal failure, multiple myeloma , and C-reactive protein. The latter group, with abnormal serum proteins, were studied to ensure that the lectin binding to AP isoenzymes was not altered by large amounts of other proteins in the serum. In 97 of 120 cases, the observed and expected bone and liver activities were within 5 U/L of each other. All recoveries were within 15 U/L of expected, with the 50:50 (recovery 8) and 50:50 (recovery 10) admixtures for myeloma serum representing the worst performance. The evidence supports that the electrophoretic conditions are adequate to reliably separate the various AP isoenzymes.

Reference ranges for bone and liver I AP isoenzymes were established on 106 females age 17 to 72 and on 107 males age 20 to 73. Results are shown in figures 4 - 7. Data obtained on males shows no significant difference between age groups for either bone or liver I AP as determined by the Mann-Whitney U test ($p = 0.1357$ and 0.2090 respectively). Data obtained on females showed no significant difference between age groups 20-29, 30-39, or 40-49 for bone AP ($P > 0.0764$). In females, a significant

Table 4 Recovery Study Summary

RECOVERY STUDY	RATIO	LIVER I U/L	LIVER II U/L	BONE U/L	INTEST U/L
High Bone/	75:25	53 (46)	--	79(86)	10(10)
High Liver	50:50	85 (86)	--	62(60)	8(9)
	25:75	105(101)	--	39(37)	4(10)
Normal Bone/	75:25	136(138)	--	36(34)	--
High Liver	50:50	121(121)	--	39(34)	--
	25:75	107(105)	--	37(39)	--
High Bone/	72:25	66(70)	16(18)	97(91)	--
Normal Liver	50:50	56(65)	27(26)	60(52)	--
	25:75	46(49)	30(31)	31(27)	--
Dialysis/	75:25	38(40)	25(24)	38(40)	--
Normal	50:50	25(24)	29(21)	28(33)	--
	25:75	63(61)	23(21)	19(23)	--
Dialysis/	75:25	100(104)	27(27)	79(77)	2(0)
High Bone	50:50	101(102)	19(19)	109(110)	2(0)
	25:75	104(101)	10(16)	120(118)	18(18)
High Bone/	75:25	33(29)	---	50(53)	14(15)
Normal	50:50	86(82)	---	90(92)	17(19)
	25:75	165(164)	---	112(105)	11 (0)
High Liver/	75:25	123(118)	---	26(31)	--
Normal	50:50	98 (96)	---	30(32)	--
	25:75	76(76)	---	31(31)	--
High IgM/	75:25	134(127)	20(35)	54(55)	--
High Liver	50:50	115(100)	33(39)	47(56)	--
	25:75	98(95)	48(48)	37(40)	--
High IgM/	75:25	111(103)	---	83(79)	16(4)
High Bone	50:50	74(73)	---	97(97)	5(7)
	25:75	42(45)	---	106(101)	8(9)
High IgG/	75:25	45(52)	---	81(81)	16(29)
High Bone	50:50	47(32)	---	93(90)	12(20)
	25:50	30(26)	---	102 (108)	12(10)
High IgG/	75:25	84(70)	15(23)	58(48)	14(21)
High Liver	50:50	83(93)	29(27)	49(49)	9 (0)
	25:75	48(50)	32(33)	23(25)	3 (0)
High IgG/	75:25	77(69)	---	60(64)	17(20)
High Bone	50:50	44(46)	---	42(43)	14(11)
	25:75	68(68)	---	83(79)	33(37)

High IgG/	75:25	106(104)	28(27)	23(27)	--
High Liver	50:50	87(96)	42(39)	39(34)	--
	25:75	65(72)	54(58)	56(46)	--
High IgG/	75:25	108(109)	4(3)	33(32)	--
Biliary	50:50	106(106)	10(12)	33(33)	--
	25:75	105(103)	15(12)	32(38)	--
High IgM/	75:25	35(32)	---	78(81)	--
Normal	50:50	44(46)	---	54(52)	--
	25:75	48(48)	---	37(37)	--
High Intest/	75:25	51(53)	6(6)	52(53)	44(41)
High Liver	50:50	71(70)	4(8)	50(55)	30(29)
	25:75	97(108)	19(20)	42(43)	15 (2)
High Intest/	75:25	36(31)	---	72(76)	44(45)
High Bone	50:50	44(45)	---	86(85)	31(31)
	25:75	51(48)	---	104(108)	15(14)
Cord Blood/	75:25	46(48)	6(6)	50(50)	41(39)
High Liver	50:50	69(67)	10(8)	47(53)	30(28)
	25:75	95(93)	19(20)	41(42)	15(14)
CRP 32 mg/dl/	75:25	90(90)	---	17(17)	---
Normal	50:50	78(76)	---	20(22)	---
	25:75	68(67)	---	20(21)	---
CRP 16 mg/dl/	75:25	86(87)	---	40(39)	---
Normal	50:50	75(76)	---	40(39)	---
	25:75	65(65)	---	38(38)	---

* Expected activity is listed with observed activity in parentheses.

Figure 4

Bone alkaline phosphatase data obtained on 107 apparently healthy males ages 20-73 years.

Figure 4

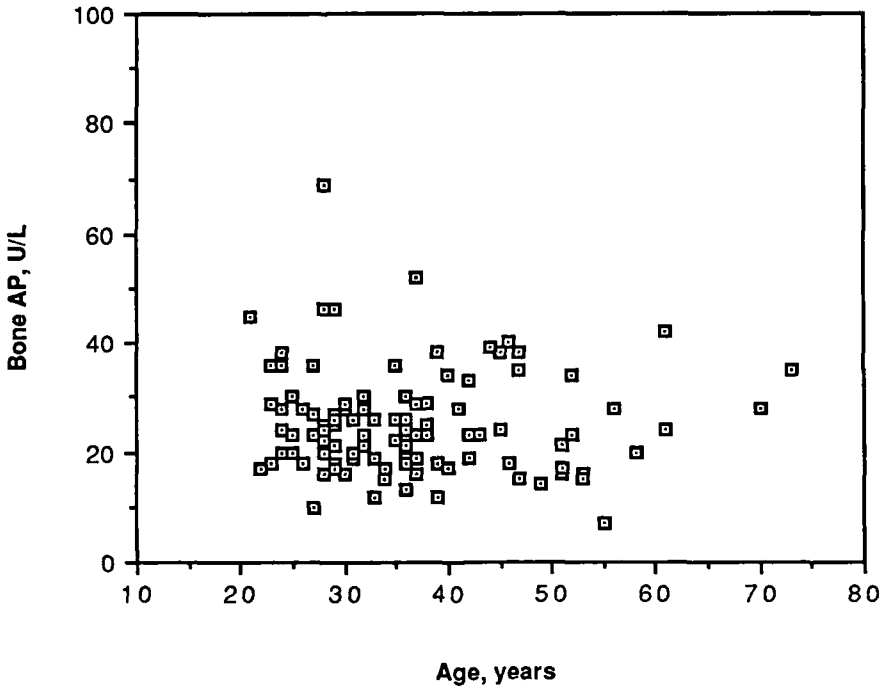


Figure 5

Bone alkaline phosphatase data obtained on 106 apparently healthy females ages 17-72 years. The open data points are pre-menopausal women. The closed data points are post-menopausal women.

Figure 5

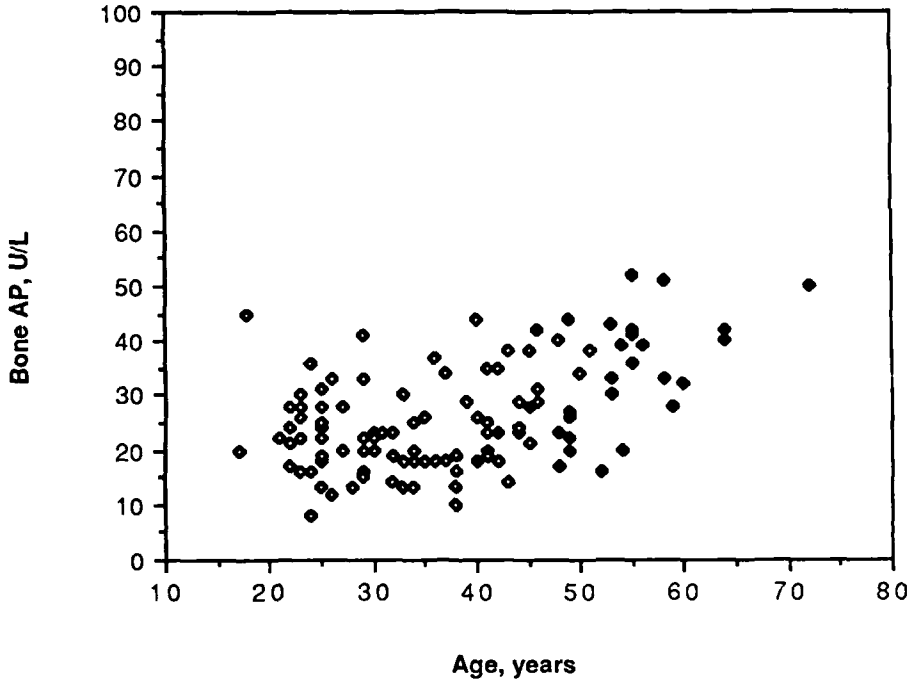

Bone AP, U/L
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Age, years

Figure 6

Liver alkaline phosphatase data obtained on 107 apparently healthy males ages 20-73.

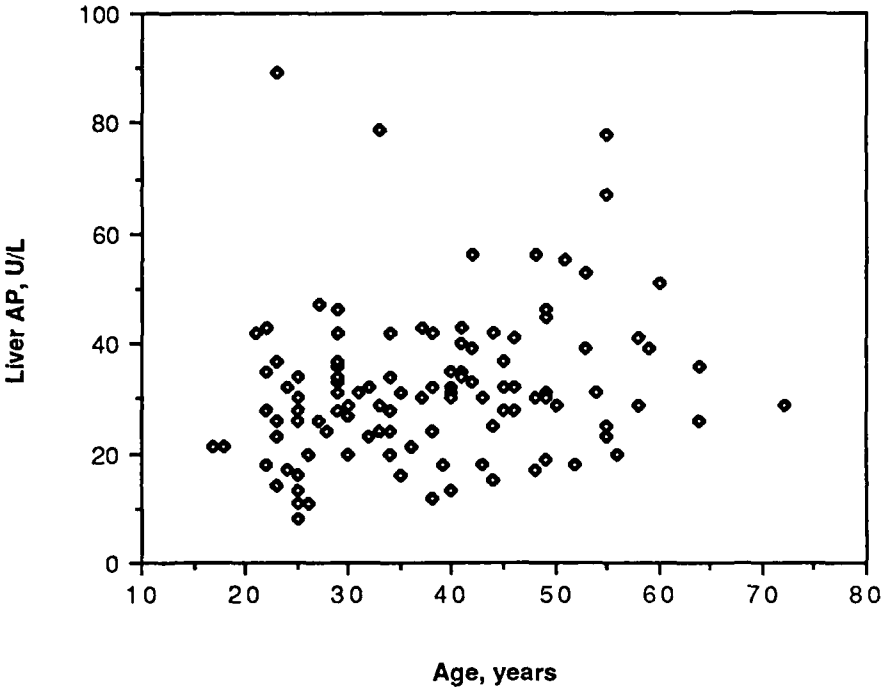
Figure 6


Liver AP, U/L
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Age, years

Figure 7

Liver alkaline phosphatase data obtained on 106 apparently healthy females ages 17-72 years

Figure 7



difference exists for bone AP between the < 50 and ≥50 age groups (p= 0.0192). For liver I, there was no significant difference between age groups for women (p = 0.0918). In addition, no significant difference was found between males and females for liver AP (p = 0.2483) and bone AP between males age 20-73 and females age 17-49 (p = 0.1056). There was a significant difference for bone AP between females ≥50 years of age and males of all age groups (p = 0.0013).The central 95% reference limits for liver I isoenzyme for adult males and females is 12-64 U/L, and for bone isoenzyme for adult males and females 17-49 years of age is 12-45 U/L. The reference range observed for bone AP for females ≥50 years of age is 16-52 U/L. Intestinal AP was found in 29% of the women ranging from 3 to 19 U/L and in 34% of the men ranging from 4 to 21 U/L. Biliary isoenzyme was not present in these apparently healthy individuals.

Comparison between WGL electrophoresis and PAGE was conducted on 71 patients who had AP isoenzymes requested by physicians. The PAGE method had been used routinely for nine years at the time of this study. The WGL method is a quantitative method reported in U/L; whereas the PAGE is a semiquantitative method which is reported as normal, slightly elevated, moderately elevated, or substantially elevated. Because of this, results from the WGL method are qualitatively compared to results from PAGE in Figures 8 - 10. The results of the 71 patients obtained on PAGE were

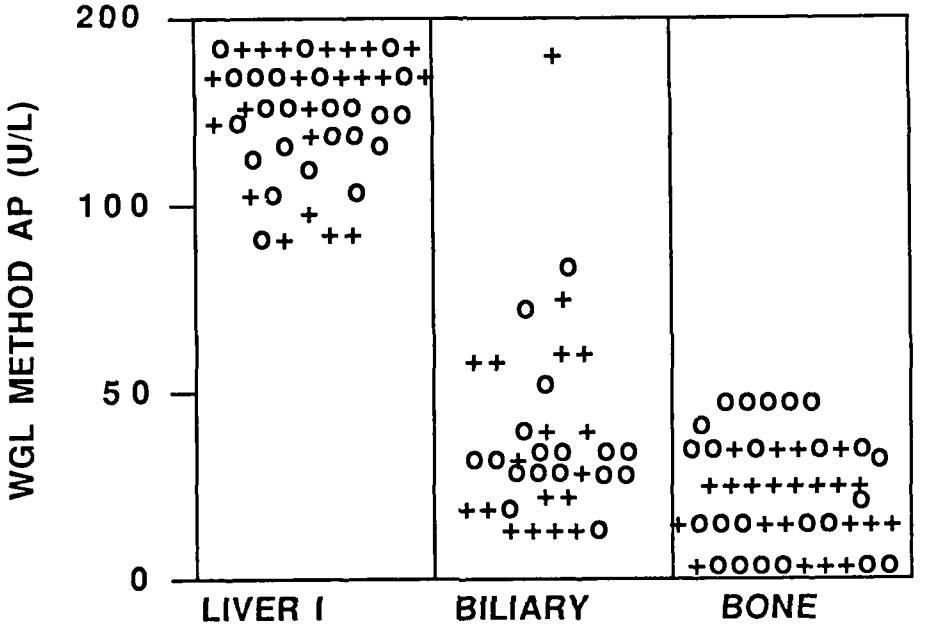
grouped into 3 categories: category 1 contained results reported as "elevated liver, normal bone"; category 2 - "normal liver, elevated bone"; category 3 -"elevated liver, elevated bone". Each category is shown as a separate graph with the X-axis divided into the three isoenzyme bands and the WGL result in U/L on the Y-axis.

Seven patients had normal liver and bone isoenzymes by PAGE. One male patient in this group had an elevated liver I isoenzyme (76 U/L) and normal bone isoenzyme (39 U/L) on WGL electrophoresis. A review of his chart showed an elevated ALT (69 U/L) which is consistent with the elevated liver I AP. Figure 8 shows that 46 patients had elevated liver I and normal bone by both methods. Thirty five of the 46 patients showed biliary isoenzyme on PAGE and on WGL electrophoresis. There was 100% agreement between methods for this group of patients. For patients with "normal liver, elevated bone" by PAGE, Figure 9, the bone fraction of one patient reported as "slightly elevated" on PAGE was normal (43 U/L) when run on WGL electrophoresis. This patient had a total AP of 93 U/L and no bone pathology was reported in her medical history. The WGL result is probably correct since it is difficult to estimate slight elevations in bone isoenzyme by PAGE. Five patients had elevated liver I, and elevated bone isoenzyme on PAGE. Figure 10 shows that on WGL all 5 patients had elevated liver I isoenzyme but one male patient had bone isoenzyme (40 U/L) within the reference interval for WGL.

Figure 8

Results of wheat germ lectin electrophoresis versus polyacrylamide gel electrophoresis of 46 patients with elevated liver and normal bone alkaline phosphatase. Males are represent by (+) and females by (o).

Figure 8

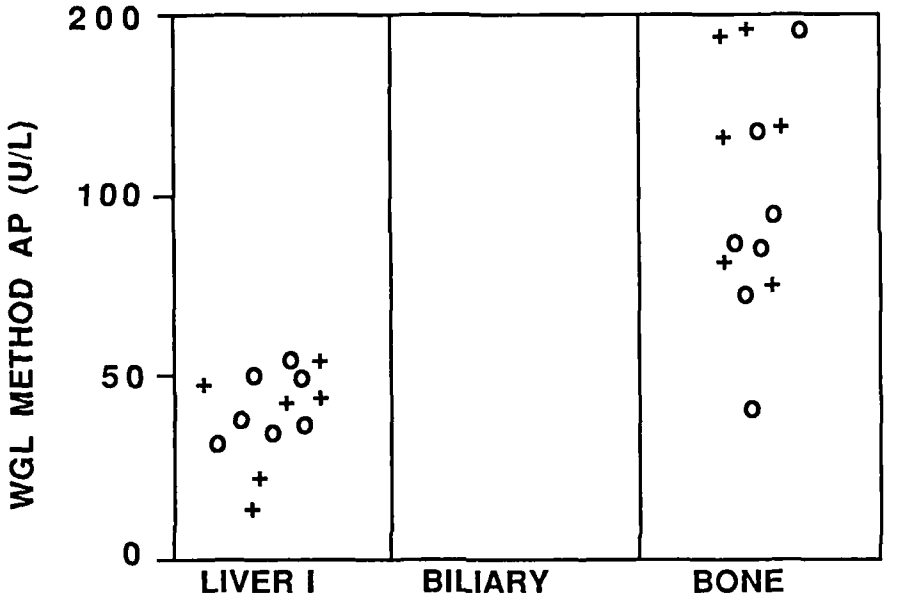


POLYACRYLAMIDE GEL ELECTROPHORESIS
(Elevated liver, Normal bone)

Figure 9

Results of wheat germ lectin electrophoresis versus polyacrylamide gel electrophoresis of 13 patients with normal liver and elevated bone alkaline phosphatase. Males are represented by (+) and females by (o).

Figure 9



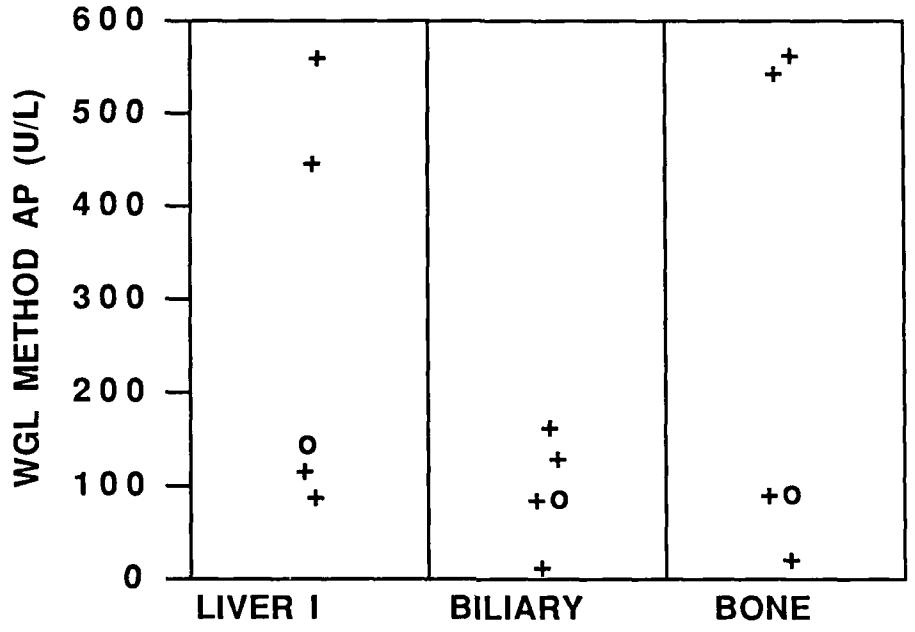
POLYACRYLAMIDE GEL ELECTROPHORESIS

(Normal liver, Elevated bone)

Figure 10

Results of wheat germ lectin electrophoresis versus polyacrylamide gel electrophoresis of 5 patients with elevated liver and elevated bone alkaline phosphatase. Males are represented by (+) and females by (o).

Figure 10



POLYACRYLAMIDE GEL ELECTROPHORESIS

(Elevated liver, Elevated Bone)

In this case, the PAGE result was reported as "slightly elevated bone". Considering the inherent uncertainties in the PAGE categorizations, the qualitative agreement between methods is excellent.

AP isoenzyme results were correlated with clinical situation for 210 patients selected from routine chemistry profiles based on elevated total AP (Table 5). Each patient's medical record was reviewed to determine diagnosis. Table 6 presents the results for 131 of the 210 patients who fell into several distinct diagnostic categories. The isoenzyme patterns are in excellent agreement with those expected based on the patho-physiology involved. Forty two patients had liver disease with all 42 showing elevated liver I isoenzyme and bone AP within the reference limits. Forty of these patients had biliary AP present. Thirty five patients had a diagnosis of some form of carcinoma or leukemia. All 35 patients had elevated liver I fractions and 29 had biliary AP present consistent with liver involvement. One patient with malignant bone cancer also had an elevated bone fraction. Nineteen patients had various cardiac diseases. Liver I isoenzyme was elevated in 17 of these cardiac patients probably reflecting decreased organ perfusion. The same 17 patients had bone AP within the reference limits. Two post heart transplant patients had elevated bone AP presumably due to damage to the ribs and sternum during surgery. Twenty three of 25 patients with renal failure had liver I

Table 5 AP ISOENZYME CLINICAL CORRELATION USING WGL

DIAGNOSIS	SEX	AGE	AP	LVR 1	LVR 2	BONE	INT
Abdomenal Aneurysm	M	74	137	77	0	37	23
Abdomenal Surgery	F	57	221	141	51	29	0
Abdomenal Trauma	M	24	220	112	88	21	0
Abdom. Wound/Gunshot	F	48	255	115	84	56	0
Abdom. Wound/Gunshot	M	40	179	150	16	13	0
Altered Mental Status	M	39	1683	1650	0	33	0
Anemia, Chronic Dis.	F	82	220	156	27	37	0
Arthritis, Rheumatoid	M	70	79	26	0	53	0
Bone Marrow Trans.	M	37	243	51	100	92	0
Bowel Obstruction	F	62	229	169	21	39	0
Bronchitis, Chronic	F	50	135	93	0	28	14
Burns, 2nd Degree	F	51	160	117	0	43	0
Carcinoma	F	50	186	130	19	37	0
Carcinoma	M	69	379	277	68	34	0
Carcinoma	F	60	267	147	0	43	77
Carcinoma, Bone	F	43	171	99	0	72	0
Carcinoma, Breast	F	73	798	710	56	32	0
Carcinoma, Cervix	F	75	413	211	0	43	0
Carcinoma, Cervix	F	61	458	430	0	28	0
Carcinoma, Colon	F	59	188	134	18	36	0
Carcinoma, Colon	F	59	207	143	21	43	0
Carcinoma, Colon	M	72	3223	2708	483	32	0
Carcinoma, Colon	F	67	243	170	29	43	0
Carcinoma, Colon	M	64	592	314	225	53	0
Carcinoma, Kidney	F	49	190	141	13	36	0
Carcinoma, Liver	M	41	259	207	30	22	0
Carcinoma, Liver	M	80	129	101	21	7	0
Carcinoma, Lung	M	45	200	150	36	14	0
Carcinoma, Lung	M	50	312	269	0	43	0
Carcinoma, Lung	M	40	310	276	15	19	0
Carcinoma, Lung	F	45	1011	930	51	30	0
Carcinoma, Lung	F	62	202	150	30	22	0
Carcinoma, Lung	M	56	421	349	47	25	0
Carcinoma, Maxilla	F	57	165	95	26	44	0
Carcinoma, Mouth	M	69	234	143	61	30	0
Carcinoma, Ovary	F	67	288	245	17	26	0
Carcinoma, Pancrease	M	73	160	94	0	38	28
Carcinoma, Rectum	M	74	175	123	14	33	5
Carcinoma, Squamous	F	51	183	152	15	16	0
Cellulitis, Groin	F	58	143	78	0	55	10
Cerebral Vas. Disease	F	56	158	97	0	51	10
Dehydration	F	61	283	179	62	42	0
Diabetes	M	50	130	75	0	29	26
Diabetes	F	65	337	301	0	22	14
Diabetes	M	48	210	170	0	40	0
Diabetes	M	43	302	275	0	27	0

Diabetes	F	52	636	585	0	51	0
Diabetes	F	65	258	204	0	54	0
Diabetes	M	41	132	54	0	78	0
Diabetes	M	50	160	125	0	35	0
Diabetes	M	50	388	316	39	33	0
Diabetes	F	35	241	224	0	17	0
Diabetes	F	63	260	226	0	34	0
Fracture, Pelvis	M	68	1560	62	0	1420	78
Fracture, Spine	M	23	172	65	0	107	0
Fracture, Tibia	F	44	159	28	0	103	27
Fracture, Tibia	M	30	176	47	0	111	18
Fracture, Pelvis	M	23	298	63	0	119	116
Gastric Obstruction	F	58	214	201	0	13	0
Heart, CHF	M	46	161	130	0	31	0
Heart, CHF	F	57	136	113	0	23	0
Heart, CHF	F	65	205	176	0	29	0
Heart, CHF	M	69	135	86	11	38	0
Heart, CVA	F	87	191	143	0	48	0
Heart, Valve Disease	M	71	218	98	0	105	15
Heart, Angina Pect.	M	54	139	103	0	36	0
Heart, Cardiac Arrest	M	68	186	157	16	14	0
Heart, Cardiac Arrest	M	46	233	110	44	40	0
Heart, Catherization	M	64	165	142	0	23	0
Heart, Artery Disease	F	52	159	119	0	40	0
Heart, Artery Disease	F	68	225	158	0	56	11
Heart, M.I.	F	84	167	109	0	58	0
Heart, M.I.	M	48	140	113	0	28	0
Heart, M.I.	F	66	378	340	0	38	0
Heart, Transplant	M	55	135	55	0	62	18
Heart, Transplant	M	34	158	54	0	65	39
Heart, Arrythmia	F	74	266	159	67	40	0
Heart, Unstab. Angina	M	46	151	113	0	38	0
HIV	M	44	152	78	49	26	0
HIV/Pneumonia	M	36	235	209	0	26	0
Hypercalcemia	M	52	85	13	0	72	0
Hypercalcemia	M	65	161	79	0	55	27
Hypercalcemia	F	45	526	287	182	57	0
Hypertension	F	31	127	87	0	40	0
Hyperthyroid	F	69	217	58	0	121	38
Hypokalemia	F	56	173	97	0	45	31
Hypokalemia	F	54	162	111	0	51	0
Hypotension	M	63	184	134	18	31	0
Oral Surgery	M	34	354	188	0	113	53
Intracranial Bleeding	M	66	84	39	32	13	0
Leukemia	M	68	370	274	74	22	0
Leukemia	M	48	169	139	12	18	0
Leukemia, Lymphocytic	F	74	298	252	26	20	0
Leukemia, Myelocytic	F	60	126	90	12	21	0
Leukemia, Myelocytic	F	39	339	241	78	20	0
Leukemia, Myelocytic	M	58	208	158	15	35	0
Liver, Ascites	F	64	303	185	79	39	0
Liver, Cirrhosis	M	39	637	497	89	51	0

Liver, Cirrhosis	M	56	618	457	136	25	0
Liver, Cirrhosis	F	58	248	181	50	17	0
Liver, Bili. Fistula	F	63	270	151	68	51	0
Liver, Cholangitis	M	47	1206	952	217	36	0
Liver, Cholangitis	F	66	390	258	70	62	0
Liver, Cholangitis	F	40	151	73	56	22	0
Liver, Cholestasis	M	71	291	236	35	20	0
Liver, Cholestasis	M	75	217	176	11	30	0
Liver, Cirrhosis	M	53	1014	781	304	30	0
Liver, Cirrhosis	M	46	634	589	31	14	0
Liver, Cirrhosis	M	41	332	208	104	20	0
Liver, Cirrhosis	M	37	311	193	87	31	0
Liver, Cirrhosis	F	56	219	163	45	10	0
Liver, Cirrhosis	F	61	174	97	19	58	0
Liver, Cirr. Early	M	54	128	95	0	33	0
Liver, Cirr. Alcohol	F	38	316	243	63	19	0
Liver, Cirr. Alcohol	M	73	326	228	65	33	0
Liver, Cirr. Alcohol	M	42	356	292	43	21	0
Liver, Cirr. Alcohol	F	42	834	567	217	50	0
Liver, Cirr. Alcohol	F	46	219	142	27	50	0
Liver, Cirr. Alcohol	F	36	162	79	41	42	0
Liver, Failure	M	48	173	138	0	35	0
Liver, Failure	F	77	152	118	11	23	0
Liver, Failure	M	50	237	188	30	19	0
Liver, Failure	F	52	367	213	110	51	0
Liver, Failure	F	36	177	97	29	51	0
Liver, Failure	M	44	688	466	261	21	0
Liver, Failure	F	47	346	249	73	24	0
Liver, G.I. Bleeder	M	71	150	69	44	36	0
Liver, Abscess	M	31	210	164	8	38	0
Liver, Hepatitis	F	86	170	116	32	22	0
Liver, Hepatitis	M	30	164	108	22	34	0
Liver, Hepatitis	M	70	150	105	21	24	0
Liver, Hepatitis	F	45	319	188	77	54	0
Liver, Hepatitis	M	45	207	143	43	21	0
Liver, Hepatitis	M	69	154	114	12	28	0
Liver, Jaundice	M	62	285	180	62	43	0
Liver, Non A, Non B	M	56	452	380	36	36	0
Liver, Obstruc. Jaund	M	34	398	310	42	41	46
Liver, Obstruc. Jaund	M	58	573	400	103	34	0
Lung, Lesion	F	76	151	106	0	45	0
Lung, Mass	M	41	197	156	0	41	0
Lung, Asthma	M	45	141	100	0	41	0
Lung, Asthma	M	68	150	110	0	40	0
Lymphoma, Large Cell	F	37	132	95	0	37	0
Malabsorption	M	56	170	71	0	58	41
Metabolic Acidosis	M	75	151	109	0	42	0
Multiple Myeloma	F	54	162	134	15	13	0
Muscular Degeneration	M	29	126	78	0	37	11
Necrosis, Hand Lesion	F	50	193	160	0	33	0
Necrosis, Lesion	M	52	201	179	0	22	0
Necrotic Tissue	M	50	219	127	0	26	66

Nontoxic Goiter	F	75	164	123	0	41	0
Osteoporosis	F	55	122	35	0	87	0
Pancreatic Ulcer	M	43	133	69	0	36	29
Pancreatitis	F	44	150	111	0	39	0
Pancreatitis	M	39	172	134	12	26	0
Pancreatitis	M	39	434	339	69	26	0
Pancreatitis	M	53	154	88	0	42	24
Pancreatitis, Acute	M	35	250	181	9	50	0
Pelvic Inflam Disease	F	25	267	231	21	15	0
Peridonitis, Chronic	F	44	164	127	0	37	0
Phlebitis	F	61	171	132	0	39	0
Phlebitis	F	79	162	128	0	34	0
Pleural Effusion	M	32	119	44	0	44	30
Pleural Effusion	F	45	245	213	0	32	0
Pneumonia	M	54	189	157	0	32	0
Pregnant, Bi-gesti	F	27	162	134	0	28	0
Pregnant,Intrauterine	F	18	216	181	0	35	0
Pregnant, Pre-term	F	28	317	301	0	16	0
Rectal Abscess	F	33	159	135	0	24	0
Renal Failure	M	50	156	34	0	100	22
Renal Failure	F	41	223	42	0	134	47
Renal Failure	F	43	293	26	0	243	23
Renal Failure	M	35	1068	32	0	876	160
Renal Failure	M	55	328	75	0	203	49
Renal Failure	F	53	265	16	0	199	50
Renal Failure	F	34	218	33	0	144	41
Renal Failure	F	29	194	58	0	105	31
Renal Failure	M	30	355	103	0	178	74
Renal Failure	M	42	178	28	0	101	50
Renal Failure	F	40	209	28	0	148	33
Renal Failure	F	42	154	40	0	99	15
Renal Failure	M	40	200	12	0	150	38
Renal Failure	F	60	131	28	0	77	26
Renal Failure	M	34	186	24	0	149	13
Renal Failure	F	65	158	38	0	107	13
Renal Failure	M	57	273	14	0	218	41
Renal Failure	M	39	153	38	0	110	5
Renal Failure	F	39	153	25	0	115	13
Renal Failure	M	59	954	48	0	763	143
Renal Failure	F	39	149	48	0	101	0
Renal Failure	F	60	194	39	0	128	27
Renal Failure	M	50	152	35	0	117	0
Renal, Nephritis	M	28	170	49	0	90	31
Sarcoma	F	52	193	112	0	50	31
Seizures	F	49	131	111	0	20	0
Seizures, Epileptic	M	50	137	107	0	30	0
Thrombocytopenia	M	22	258	191	41	26	0
Thrombocytopenia	M	42	205	90	18	76	18
Trauma, Brain/Skull	M	24	1454	612	135	747	0
Trauma, Cerebral	M	20	206	19	0	187	0
Trauma, Cerebral	F	56	137	90	0	47	0
Trauma, Cerebral	M	26	1760	123	0	1152	475

Trauma, Cranial Bleed	F	74	153	61	0	47	45
Trauma, Internal	M	59	236	158	0	78	0
Trauma, Internal	F	46	391	293	74	24	0
Trauma, Internal	M	22	513	421	62	30	0
Trauma, Intern/Fract	M	66	510	286	0	138	86
Trauma, Fractures	M	26	201	49	0	125	27
Tuberculosis	F	47	378	261	68	49	0
Urosepsis, Uremia	F	88	160	32	0	90	38

Table 6 Clinical Correlation n= 131

Diagnosis	n	LVR I		Biliary	Bone		Intest
		Elev	Norm		Elev	Norm	
Liver Disease	42	42	0	40	0	42	1
Carcinoma	35	35	0	29	1	34	1
Cardiac Disease	19	17	2	4	2	17	4
Renal Disease	25	2	23	0	25	0	23
Bone Disease	7	0	7	1	7	0	5
Hyper-Calcemia	3	2	1	1	3	0	0

AP within reference limits. All 25 patients had elevated bone AP with 23 of these patients also showing intestinal isoenzyme. No biliary isoenzyme was present. Seven patients had elevated total AP due to a variety of bone pathologies. Five patients had bone fractures. One patient had osteoporosis and another had undergone a bone marrow transplant. All 7 patients showed elevated bone AP consistent with osteoblastic activity in these diseases. All patients had normal liver I isoenzyme activity and one had biliary isoenzyme present. Three patients with hypercalcemia had elevated bone AP. Two of these patients had elevated liver I AP and had concurrent transaminase elevations.

Placental AP begins to rise in pregnancy toward the end of the first trimester and peaks in the third trimester (25). Placental AP is heat stable; whereas, bone and liver I isoenzymes are completely destroyed when heated at 56°C for 30 minutes (4). The sera of 3 pregnant women were incubated at 56°C for 30 minutes prior to WGL isoenzyme assay on a heated and an unheated aliquot. The residual placental AP co-migrated with the liver I isoenzyme in this electrophoretic procedure.

DISCUSSION

The commom serum AP isoenzymes are clearly separated and easily quantitated using agarose electrophoresis containing WGL. The substrate, 4-methyl umbelliferyl phosphate, permits detection of the activities found in healthy individuals with good precision and produces a linear response to highly pathological activities. Moderately increased activities of liver or bone AP can be quantitated with good precision.

Separation of isoenzymes is dependent on the quality of WGL used in the procedure. Two lots of WGL obtained from Sigma and one lot from Calbiochem, La Jolla, Ca. 92037 gave equivalent performance as reported here. However, inadequate separation was seen with one lot from Sigma and one lot from Boehringer Mannheim, Mannheim, FRG. Wheat germ lectin preparations can have variable performance and new lots must be pre-tested in parallel with an acceptable lot for suitable separation of the isoenzymes. We have found that frozen aliquots of human serum pools are satisfactory for this purpose and for use as daily control materials.

The separation of AP isoenzymes requires that the buffer pH be maintained at 8.9 $\pm$0.1. Electrophoresis

conducted using trizma/barbital/sodium barbital buffer above pH 9.3 showed increases in the isoenzyme fractions normally migrating at the liver I and intestinal isoenzyme positions, with concomittant decreases in the bone fraction. This change in apparent isoenzyme distribution probably reflects decreased binding to the bone isoform which alters it's migration during electrophoresis.

The bone fraction accounted for approximately 50% of the total AP activity in females in the $\leq$49 age groups and males of all age groups. This conflicts with Kuwana, et al., (43) who found that the bone isoenzyme represented approximately 70% of the total AP. Kuwana and co-workers, instead of incorporating WGL into agarose, soaked cellulose acetate in a lectin solution. In women age $\geq$50 years, all of whom were postmenopausal, bone isoenzyme represented a higher percentage of the total AP than in younger women. This higher bone AP activity agrees with the results obtained by Denny, et al., (44) using an heat inactivation technique to quantitate AP isoenzymes. The difference in activity of bone AP seen between pre-and postmenopausal women is significant and shows that the quantitation of the bone fraction using this method may be of benefit in the prediction of postmenopausal osteoporosis.

Optimum AP isoenzyme separation and quantitation is sensitive to lectin concentration, buffer pH, procedural details and the reactivity each isoenzyme has for the

substrates. Reference ranges allow clinical interpretation of results under these conditions. The ability to quantitate AP has been verified by clinical correlation studies in a variety of pathologies.

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